Cellular Cardiomyoplasty Improves Survival After Myocardial Injury

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Background—Cellular cardiomyoplasty is discussed as an alternative therapeutic approach to heart failure. To date, however, the functional characteristics of the transplanted cells, their contribution to heart function, and most importantly, the potential therapeutic benefit of this treatment remain unclear.

Methods and Results—Murine ventricular cardiomyocytes (E12.5–E15.5) labeled with enhanced green fluorescent protein (EGFP) were transplanted into the cryoinjured left ventricular walls of 2-month-old male mice. Ultrastructural analysis of the cryoinfarction showed a complete loss of cardiomyocytes within 2 days and fibrotic healing within 7 days after injury. Two weeks after operation, EGFP-positive cardiomyocytes were engrafted throughout the wall of the lesioned myocardium. Morphological studies showed differentiation and formation of intercellular contacts. Furthermore, electrophysiological experiments on isolated EGFP-positive cardiomyocytes showed time-dependent differentiation with postnatal ventricular action potentials and intact \( \beta \)-adrenergic modulation. These findings were corroborated by Western blotting, in which accelerated differentiation of the transplanted cells was detected on the basis of a switch in troponin I isoforms. When contractility was tested in muscle strips and heart function was assessed by use of echocardiography, a significant improvement of force generation and heart function was seen. These findings were supported by a clear improvement of survival of mice in the cardiomyoplasty group when a large group of animals was analyzed (n=153).

Conclusions—Transplanted embryonic cardiomyocytes engraft and display accelerated differentiation and intact cellular excitability. The present study demonstrates, as a proof of principle, that cellular cardiomyoplasty improves heart function and increases survival on myocardial injury. (Circulation. 2002;105:2435-2441.)

Key Words: transplantation • cells • electrophysiology • contractility • survival

Cardiovascular diseases are the most frequent cause of death in the western hemisphere. The critical loss of functional cardiomyocytes causes a severe deterioration of pump function, resulting in heart failure. Because differentiated cardiomyocytes lack prominent regenerative capacity, heart transplantation remains the only effective causal therapy. Because of the increasing number of patients requiring this treatment and the decline in available donor organs, alternative methods, such as cellular cardiomyoplasty,1–3 are urgently needed. Thus far, however, convincing evidence showing a clear therapeutic benefit of this approach is lacking.

To compare survival in a large group of mice after heart injury alone or combined with cellular cardiomyoplasty, we used an operative procedure with very low mortality.4 A combination of morphological, functional, and molecular methods enabled us to gain detailed insight into differentiation, physiological function, and contractility of transplanted cells and their role for heart function.

Methods

The Animal Care Committee of the University of Cologne approved all the procedures performed on animals.

Mouse Breeding and Harvesting of Embryonic Cardiomyocytes

Transgenic mice5 of the strain HIM:OF1 or C57/B16 were bred and enhanced green fluorescent protein (EGFP)-positive embry-
onic ventricular cardiomyocytes (E12.5-E15.5) harvested as reported. After dissociation, the cells were resuspended in DMEM (20 000 cells/μL). Flow cytometry was performed as described.

**Operation and Cell Injection**

Male wild-type mice of the respective strains were used as recipients. The surgical procedure and the injection of cells (100 000 cells diluted in 5 μL of solution) were performed as reported. For control, EGFP-positive cardiomyocytes or solution without cells was injected into the intact myocardium or the cryolesioned myocardium, respectively.

**Histology, Immunohistochemistry, and Western Blot Analysis**

Morphological preparation and staining of tissue samples were performed as described. Nuclei were stained with DAPI (Vector Laboratories). To evaluate cross-striation, anti-α-sarcomeric actin (1:800, Sigma) primary and Cy3 goat anti-mouse (1:1000, Rockland) secondary antibodies were used. Electron microscopy was performed according to standard protocols.

Proteins of cell lysates of cardiac and skeletal muscle were separated by 10% SDS-PAGE. The membranes were probed with anti–troponin I (TnI) antibody (clone 6F9, Dunn Labortechnik), which recognizes both TnI isoforms. Comparable protein loading was assessed with Coomassie staining.

**Electrophysiology**

Single cardiomyocytes were isolated with Langendorff perfusion at 5 or 6 (early) and 11 to 14 (late) days after operation. Transplanted cardiomyocytes were identified on the basis of their EGFP expression. Action potentials (APs) and ionic currents were recorded with square pulse voltage step to −100 mV from a holding potential of −80 mV. All experiments were performed at room temperature. For determining the inactivation kinetics of the L-type Ca²⁺ current, data were normalized, offset-corrected, and fitted with a double-exponential equation.

**Isometric Tension Measurements**

Defined tissue strips (2.0 mm long and 0.5 mm wide) were obtained under a fluorescent microscope with a self-manufactured stencil. The strips were skinned with 1% Triton X-100 as described in Reference 10, with the modification of added protease inhibitors [0.2 mmol/L PMSF, 10 μmol/L leupeptin, 10 μmol/L antipain, and 5 μg/mL aprotinin]. The strips were placed into relaxing solution (pCa 7.5) and mounted in a myograph (Scientific Instruments). Strips were prestretched by 10% of their slack length, and maximum Ca²⁺-activated force was initiated by exposure to activating solution (pCa 4.5). Experiments were performed at 37°C.

**Echocardiography**

Mice were anesthetized, and heart function was monitored on days 1, 4, 7, and 14 after operation. Echocardiography was performed with an ultrasound machine (HDI-5000, ATL-Ultrasound) equipped with a linear-array transducer (CL15-7) operating at 15 MHz and providing frame rates up to 284 Hz. Centrifuged gel was used for acoustic coupling to minimize imaging artifacts. Parasternal short- and long-axis views in B and M imaging modes were performed. In addition to wall thickness, the dimension of the cavity and the ejection fraction were calculated. Regional wall motion was visually assessed and analyzed offline. Wall motion scores were applied as suggested by the American Society of Echocardiography. Myocardial segments were subdivided by use of the 15-segment model. The reader was blinded to the study group, and analysis of the data was performed 1 month after data acquisition to minimize bias.

**Statistics**

For statistical analysis of the time course of survival, both log-rank and Breslow tests were used. The latter weights, in particular statistical, differences that occurred early during the time course. The statistical significance of other parameters was examined with Student’s t test; a value of P<0.05 after Bonferroni correction was considered significant. Wall motion scores were tested by repeated-measurements ANOVA. Data are indicated as mean±SEM (electrophysiology, echocardiography) or mean±SD (tension measurements).

**Results**

**Identification of Transplanted Cells, Engraftment, and Differentiation**

Large, reproducible lesions were induced in mouse hearts by cryoinjury. To allow direct identification of the transplanted cells, we used transgenic embryos (E12.5-E15.5) in which the cardiac α-actin promoter drives the EGFP expression (Figure 1a). Flow cytometry of E13.5 ventricular cardiomyocytes evidenced that 60% to 70% of cells (n=5) remained vital after enzymatic isolation and that >50% were cardiomyocytes (Figure 1b). To understand the consequences of heart lesion in the mouse model, van Gieson staining and electron microscopy were used. Computer-assisted evaluation of infarct size in van Gieson stains revealed that ≈43±7.0% (n=5) of the ventricular circumference was transformed into fibrous scar tissue on cryoinjury. Two days after cryoinjury, necrotic decay of cardiomyocytes within the injured area and loosening of the tissue because of edema was detected (Figure 1, c and d, top). After 4 days, only cellular debris remained, accompanied by the invasion of fibroblasts into the injured area (middle). After 6 days, distinct fibrotic scar tissue formed and de novo synthesis of stable extracellular matrix became visible (bottom). This suggests that the most mechanically unstable period occurs after the necrotic degeneration of cardiomyocytes and before the fibrotic wound healing, ie, 2 to 6 days after cryoinjury.

Because of the differences in survival observed at early stages after operation (see below), our analysis focused on the first 2 weeks after cardiomyoplasty. During the first few days after operation, the transplanted cells were round and scattered within the injured area (data not shown). After 6 days, elongation and physiological alignment of cells took place. After 2 weeks, the implanted cardiomyocytes displayed transmural engraftment in the cryolesioned area (Figure 2a). The majority of transplanted cardiomyocytes were oriented in a manner similar to that of the native muscle fibers. In fact, ventricular-like rod-shaped cardiomyocytes with end-to-side and end-to-end contacts were found (Figure 2b). The cells were mononucleated, and the EGFP expression was restricted to cardiomyocytes (Figure 2c). Differentiation was also confirmed by distinct cross-striation detected with cardiac α-actin staining (Figure 2d) and demonstration of an intact myofibrillar apparatus by electron microscopy (Figure 2e, top right). The ultrastructural analysis revealed a large amount of rough endoplasmic reticulum in the transplanted cells, indicating the metabolically activated status of the cells (Figure...
Within a few days after transplantation, intercalated disks were detected, suggesting the establishment of electromechanical coupling (Figure 2e, bottom right). The reduced size of the transplanted cardiomyocytes and the relatively thickened cross-striation suggest that the cells late after operation are not yet terminally differentiated. Taken together, our morphological studies demonstrate that the cryolesion is replaced by fibrotic scar tissue within a week.
Cellular cardiomyoplasty yields successful engraftment, physiological orientation, and differentiation of the implanted cells.

Functional Characteristics of Transplanted Cells

Next, we wondered whether the physiological characteristics of transplanted cells were similar to those of native cardiomyocytes. Because of their key role for heart function, we examined the expression of ion channels. Isolated transplanted cardiomyocytes could be easily identified because of their EGFP expression (Figure 3a) and functionally characterized with the patch-clamp technique. Early after operation, many round cells were observed, whereas late after operation, higher numbers of rod-shaped transplanted cells were seen. The time-dependent differentiation of transplanted cardiomyocytes was illustrated by comparison of the 90% AP duration (APD90) of embryonic and transplanted cardiomyocytes. Early after operation, the transplanted cells displayed spontaneous electrical activity with depolarized resting membrane potentials (56.3±0.3 mV, n=3) and prolonged APD90 (208.3±7 ms, n=3) (Figure 3c and 3e). The significant shortening of the APD90 in early-postoperation cells compared with E12.5/E13.5 embryonic cardiomyocytes (359.2±40 ms, n=5, Figure 3e) suggested differentiation. In fact, these early-postoperation cells resembled ventricular cardiomyocytes of the late embryonic/perinatal stage. At this stage, intact β-adrenoceptor–mediated modulation, a hallmark for physiological heart function, was already seen, because isoprenaline (1 μmol/L) led to prolongation of the APD and a more positive plateau phase (Figure 3c). When late-transplanted cells were measured, stable resting membrane potentials (−68±8 mV, n=8) similar to those of native cells (−71.4±0.5 mV, n=12) isolated from the same hearts were found. In control (Figure 3b) and transplanted (Figure 3d) cells, electrical stimulation evoked the typical spiky APs with short-lasting APD90s of 35.8±0.8 (n=12) and 41.9±2.4 ms (n=8) respectively (Figure 3e). I_k, critical for setting the membrane potential; therefore, current densities were determined. These were similar in wild-type (4.8±0.4 pA/pF, n=10) and late-transplanted (4.5±0.1 pA/pF, n=9) but significantly lower in early-transplanted (1.3±0.2 pA/pF, n=5) cardiomyocytes. In early- and late-postoperation cardiomyocytes, the L-type Ca²⁺ current, a key component of excitation-contraction coupling, displayed normal biophysical characteristics (Figure 3f) and similar current densities (Figure 3g, top). In line with the establishment of intact Ca²⁺-induced Ca²⁺ release during differentiation, the inactivation kinetics of the L-type Ca²⁺ current were found to be accelerated in late-transplanted cells (Figure 3g, bottom). Occasionally, a tight connection between EGFP-positive and native cardiomyocytes was seen after the isolation. When lucifer yellow (3 mg/mL) was included in the patch pipette, the passage of dye from the patched EGFP-positive cell to the tightly connected native cardiomyocyte was observed (n=3), suggesting the expression of functional gap junctions between these cells. To gain more insight into the differentiation of transplanted cells, the expression pattern of TnI, known to switch at the perinatal stage from the slow skeletal to the cardiac isoform (Figure 4a), was analyzed by Western

Figure 3. Electrophysiological characteristics of transplanted cells. a, Isolated transplanted cells are identified on the basis of their EGFP expression (note cross-striation). b through d, Current-clamp recordings in a control ventricular cardiomyocyte (b) and in an early-postoperation cardiomyocyte with intact β-adrenergic response (c). d, Current-clamp recording in a late-postoperation cell. e, Comparison of APD90 shows significant prolongation in early-embryonic and early-postoperation cells. Wild-type and late-postoperation cells display similar values. f, Voltage-clamp experiments show functional expression of Na⁺ and L-type Ca²⁺ currents in an early-postoperation cell. Na⁺ currents are evoked by 20-ms depolarizing pulses to −40 mV; L-type Ca²⁺ current-voltage behavior is obtained by applying 50-ms depolarizing pulses from −40 to 40 mV in 10-mV intervals (holding potential, −80 mV). g, Top, L-type Ca²⁺ current density in early- and late-postoperation cardiomyocytes (step potential, 0 mV). Bottom, Inactivation kinetics of I_{Ca,L} (step to 10 mV) in a late-transplanted (r_{inact}=5.2 ms) and early-transplanted (r_{inact}=27.2 ms) same cell as shown in f cardiomyocyte. 1 and 2 indicate early and late transplanted cells, respectively. Averaged data are shown as mean±SEM. Bar=20 μm.
blotting. No TnI was detected in untreated cryoinfarcted tissue strips (n=11005). Showing engraftment and start of differentiation, both the skeletal (26-kDa) and the cardiac (30-kDa) TnI isoforms were detected 5 days after operation in treated ventricular muscle strips. Interestingly, as early as 2 weeks after transplantation, an almost complete switch to the adult cardiac TnI isoform was observed, suggesting accelerated differentiation of the transplanted cardiomyocytes (Figure 4a). These experiments show that the transplanted cells are physiologically intact and differentiate faster.

Contractility and Heart Function

To investigate the functional contribution of contractile proteins, maximal force development at 30 μmol/L Ca^{2+} was determined in permeabilized muscle strips. In the tissue strips harvested from the lesioned areas without engrafted cells, almost no contractile force development (36.9±27 μN, n=5, Figure 4b) was measured. In clear contrast, a significant increase of force development (221.4±52 μN, n=5, Figure 4b) was observed when strong EGFP fluorescence confirmed engraftment of a large number of cells. When the active isometric force per cross-sectional area of tissue strips containing transplanted cells was normalized, it amounted to 10% of controls (Figure 4a, inset). To assess whether this force could be generated by the engrafted cardiomyocytes, their total number was counted in tissue slices yielding 2000 (n=4) cells, 22% of control strips. With their smaller volume taken into account, the theoretical force per cross-sectional area of tissue strips containing transplanted cardiomyocytes lies in the range of 11% of control, close to our experimentally determined value. When fibroblasts instead of embryonic cardiomyocytes were transplanted, almost no contraction (48.6±16.6 μN, n=3) was detected. To evaluate whether this recovery of contractility correlated with an improvement in vivo, echocardiographic analysis was performed. M-mode images in the region of the cryoinfarct-
transplantation demonstrated that in sham-operated mice, contractility remained low during 2 weeks of observation (Figure 5a), whereas in animals that received transplants, a clear time-dependent improvement was seen (Figure 5b). For detailed assessment of therapeutic efficacy, wall motion scores were determined in the B mode. When the wall motion scores in the anterolateral region of the left ventricle were compared, consistently bad values were found in sham-operated mice, whereas a significant improvement was seen in mice after cellular cardiomyoplasty over time (Figure 5c). When the left ventricular ejection fraction was determined 4 days after operation, no difference was noted between the untreated (28.4±5.3%, n=5) and the treated (25.2±6.6%, n=5) groups. Two weeks after operation, however, global left ventricular function was significantly improved in the cardiomyoplasty group (50.6±4.9%, n=5) versus controls (18.4±4.7%), in which a steady deterioration over time occurred (Figure 5d, P<0.01). These data show a remarkable recovery of left ventricular function after cardiomyoplasty.

Animal Survival

A main goal of this study was to clarify whether cellular cardiomyoplasty has an impact on clinical outcome. The time course of survival showed a significant (log-rank P<0.05, Breslow test P=0.02) improvement in the group with cardiomyoplasty (Figure 6). Fourteen days after operation, mortality in the treated group was reduced by almost 50% compared with the group with cryolesion but without treatment. At later time points, no further difference in survival was found.

Discussion

The present study provides compelling evidence that cellular cardiomyoplasty results in a significant improvement of postoperative survival in a large group of animals. Although other groups have previously provided indirect evidence for improved left ventricular function,2,14 our data demonstrate for the first time that transplanted embryonic cells (1) differentiate, (2) are physiologically intact, (3) partially restore contractile force, and (4) improve regional and global left ventricular performance. Prominent differences in mortality between sham-operated mice and mice with cardiomyoplasty occur at a time when the cardiomyocytes are not yet differentiated and echocardiography shows no clear functional improvement. We therefore presume that the better clinical outcome is also related to decreased compliance of scar tissue and possible beneficial effects of the injected cardiomyocytes on scar formation.15 Nevertheless, because experiments that used fibroblasts instead of embryonic cardiomyocytes did not yield any recovery of contractile function, the use of noncontractile cell types for cellular cardiomyoplasty is questionable, as suggested by other groups.2,16 The formation of intercalated disks and the passage of lucifer yellow from EGFP-positive cells to native cardiomyocytes suggest at least some degree of electrical coupling between the grafted cells and the native myocardium. Future studies should analyze this important topic in detail.

Therapeutic strategies using human embryonic cardiomyocytes for cell replacement will be limited because of ethical and immunological problems. Although grafting of autologous skeletal muscle tissue is devoid of these problems, lack of electrical coupling19 and different contractile properties20 pose considerable limitations to their therapeutic use. Therefore, the use of embryonic stem cells,21 somatic stem cells,22 and/or their in vivo transdifferentiation23 represent potential alternatives in the treatment of cardiovascular diseases, even more so because bone marrow mobilization improves repair and survival of infarcted mice.24 It remains to be proved, however, that embryonic/somatic stem cell–derived “cardiomyocytes” display typical cardiac-like functional characteristics and augment long-term survival.

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